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(54) **PHYTOESTROGEN PRODUCT OF RED CLOVER AND PHARMACEUTICAL USES THEREOF**

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(51) **Int. Cl.**

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(58) **Field of Classification Search**

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See application file for complete search history.

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(57) **ABSTRACT**

The present invention provides compositions comprising optimized ratios of Red clover phytoestrogens as determined by a proprietary physiologically based pharmacokinetic and pharmacodynamic model. The compositions are useful for modulating bone remodeling, and prevention and treatment of osteoporosis.

**10 Claims, 15 Drawing Sheets**

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Figure 1

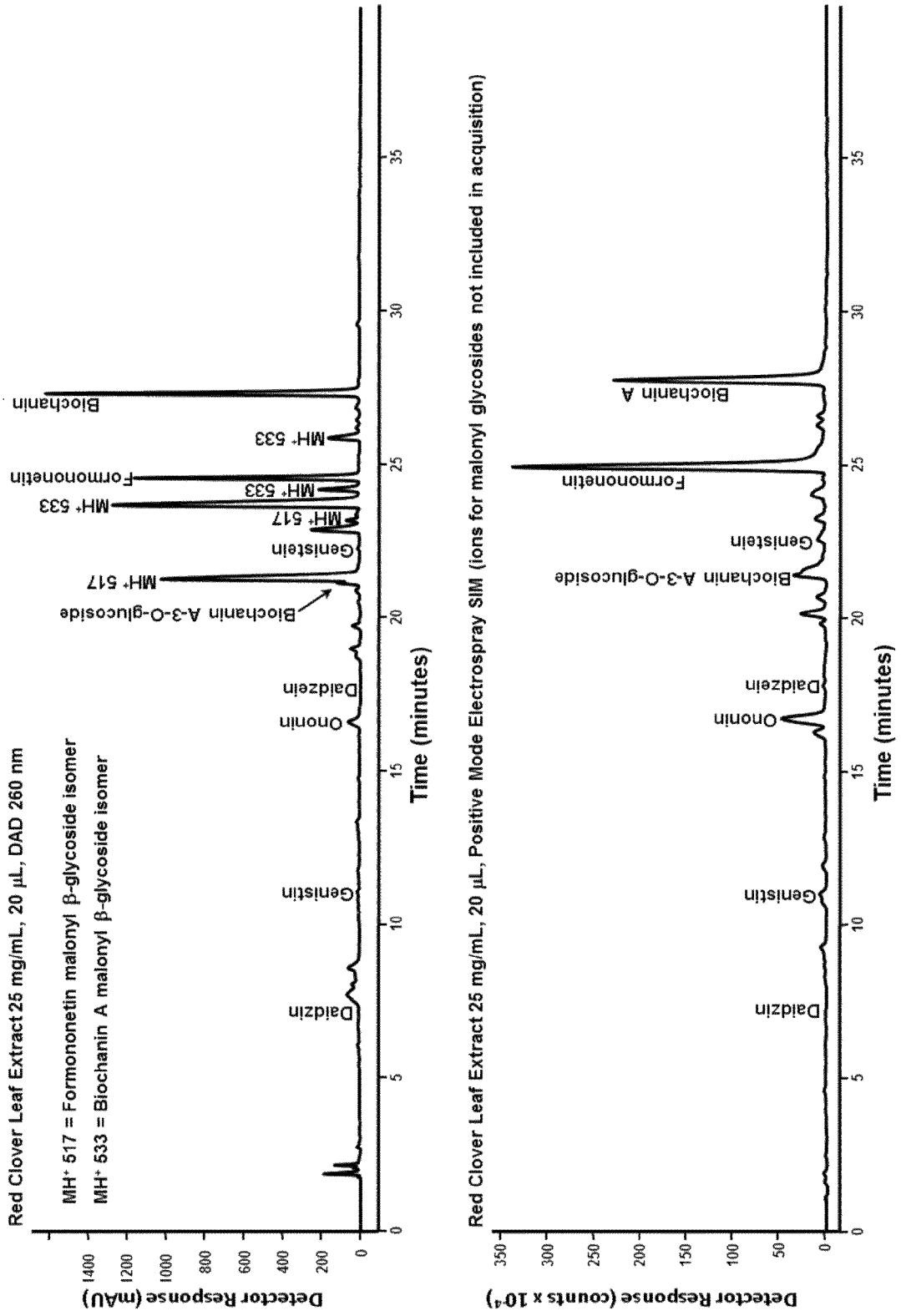

Red Clover Leaf Extract 25 mg/mL, 20 µL, DAD 260 nm
MH+ 517 = Formononetin malonyl β-glycoside isomer
MH+ 533 = Biochanin A malonyl β-glycoside isomer
Detector Response (mAU)
Time (minutes)
Daidzin
Genistin
Ononin
Daidzein
Biochanin A-3-O-glucoside
MH+ 517
Genistein
MH+ 517
MH+ 533
MH+ 533
Formononetin
MH+ 533
Biochanin
Red Clover Leaf Extract 25 mg/mL, 20 µL, Positive Mode Electrospray SIM (ions for malonyl glycosides not included in acquisition)
Detector Response (counts x 10⁴)
Time (minutes)
Daidzin
Genistin
Ononin
Daidzein
Biochanin A-3-O-glucoside
Genistein
Formononetin
Biochanin A

Figure 2

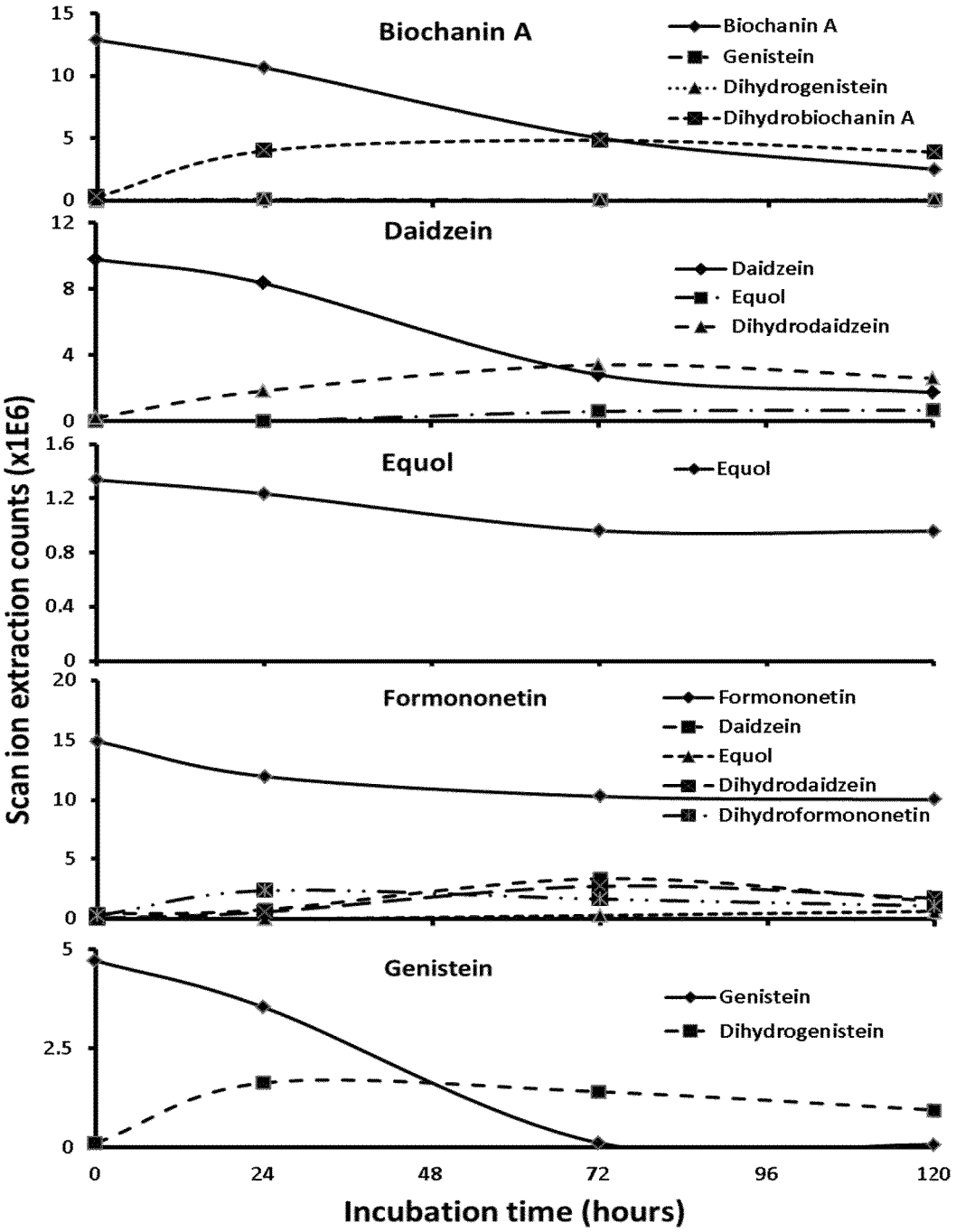

Biochanin A
Biochanin A
Genistein
Dihydrogenistein
Dihydrobiochanin A
Daidzein
Daidzein
Equol
Dihydrodaidzein
Equol
Equol
Formononetin
Formononetin
Daidzein
Equol
Dihydrodaidzein
Dihydroformononetin
Genistein
Genistein
Dihydrogenistein
Scan ion extraction counts (x1E6)
Incubation time (hours)
0
24
48
72
96
120

Figure 3

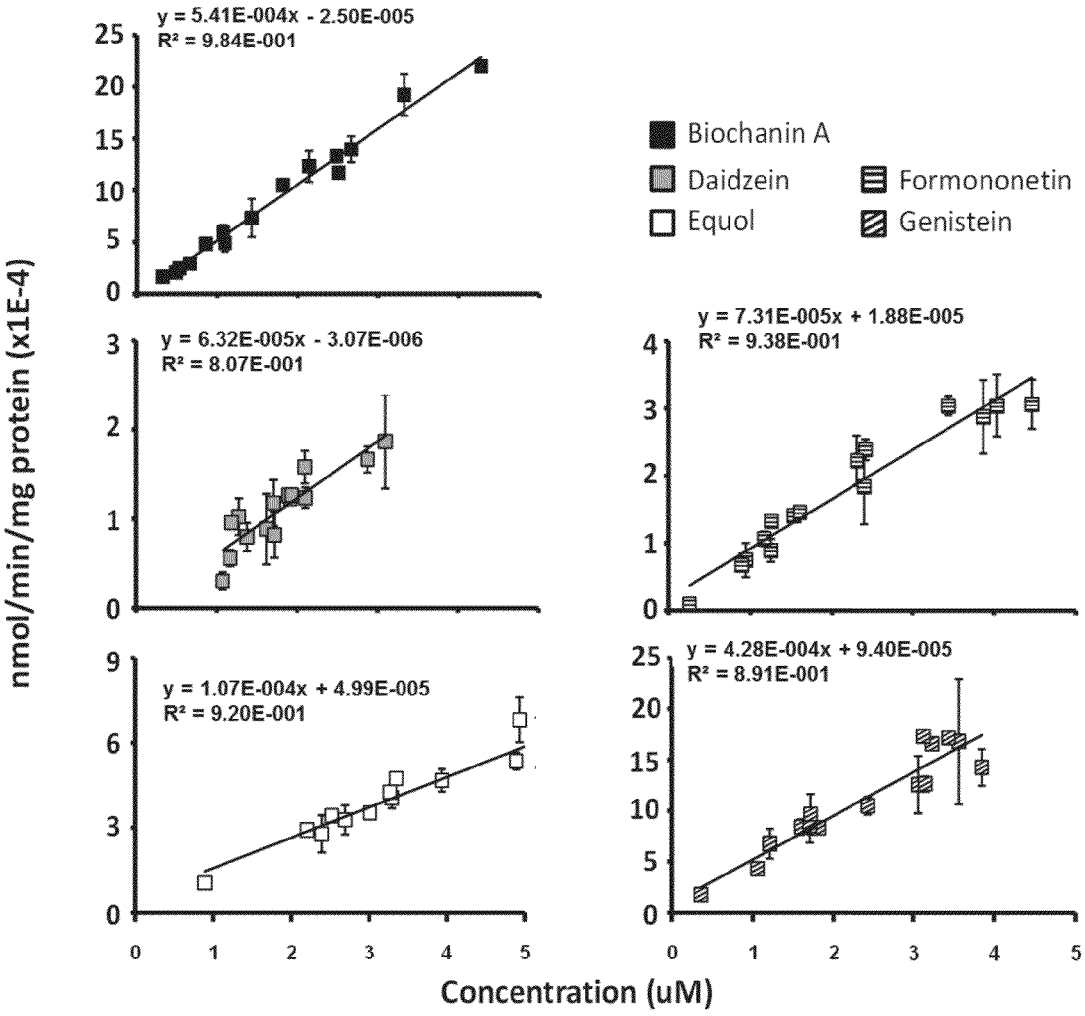

y = 5.41E-004x - 2.50E-005
R² = 9.84E-001
Biochanin A
Daidzein
Equol
Formononetin
Genistein
y = 6.32E-005x - 3.07E-006
R² = 8.07E-001
y = 7.31E-005x + 1.88E-005
R² = 9.38E-001
y = 1.07E-004x + 4.99E-005
R² = 9.20E-001
y = 4.28E-004x + 9.40E-005
R² = 8.91E-001
nmol/min/mg protein (x1E-4)
Concentration (uM)

Figure 4

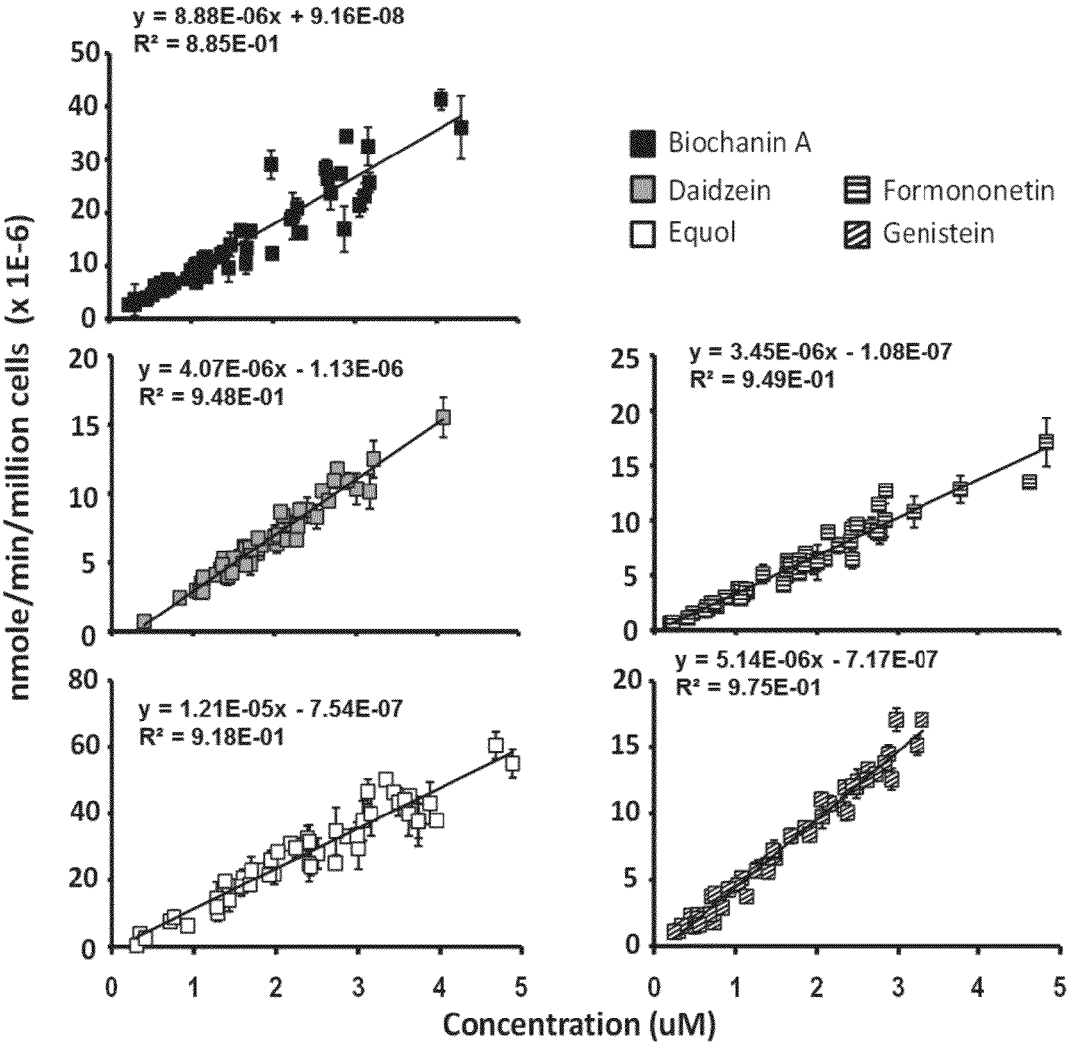

y = 8.88E-06x + 9.16E-08
R² = 8.85E-01
y = 4.07E-06x - 1.13E-06
R² = 9.48E-01
y = 3.45E-06x - 1.08E-07
R² = 9.49E-01
y = 1.21E-05x - 7.54E-07
R² = 9.18E-01
y = 5.14E-06x - 7.17E-07
R² = 9.75E-01
Biochanin A
Daidzein
Equol
Formononetin
Genistein
nmole/min/million cells (x 1E-6)
Concentration (uM)

Figure 5

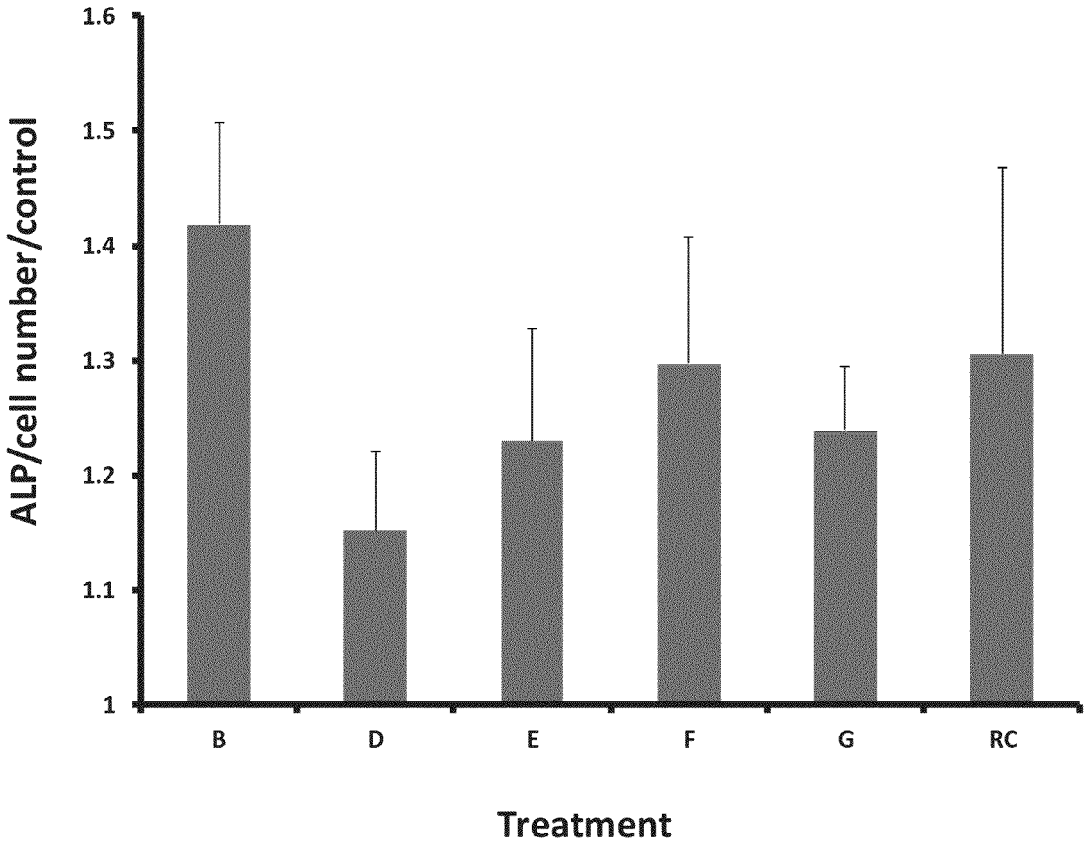


Figure 6

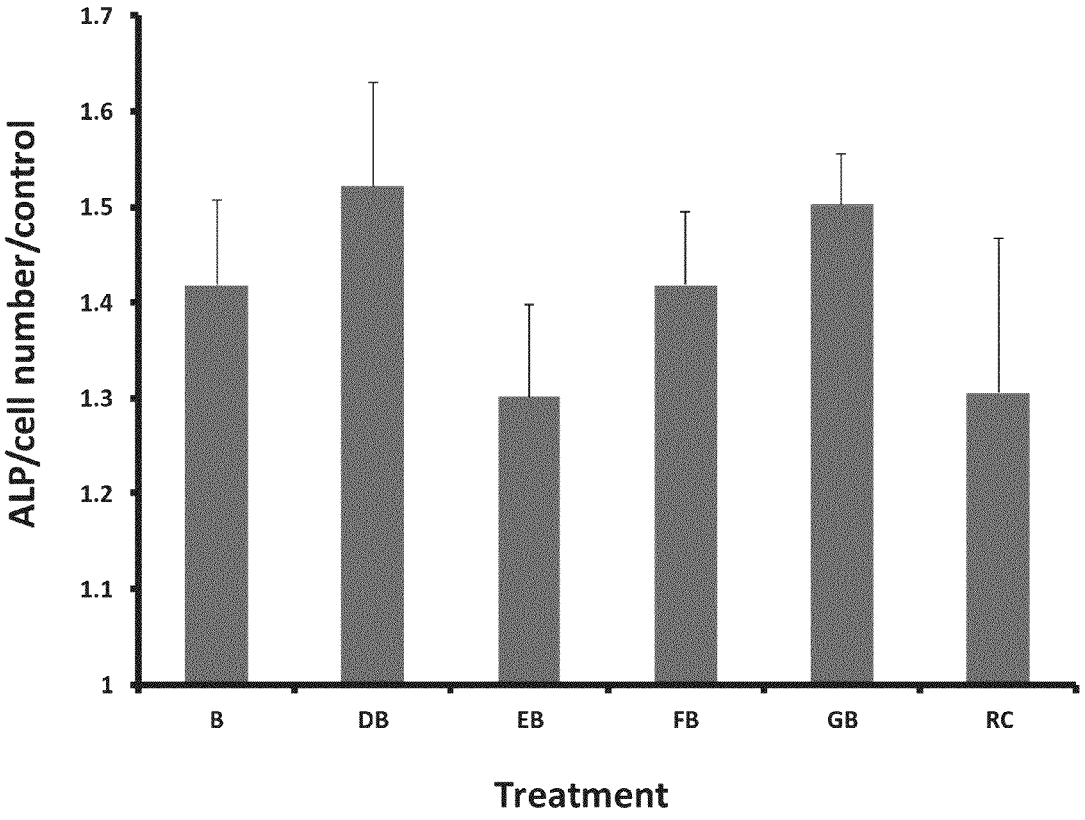

ALP/cell number/control
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
B
DB
EB
FB
GB
RC
Treatment

Figure 7

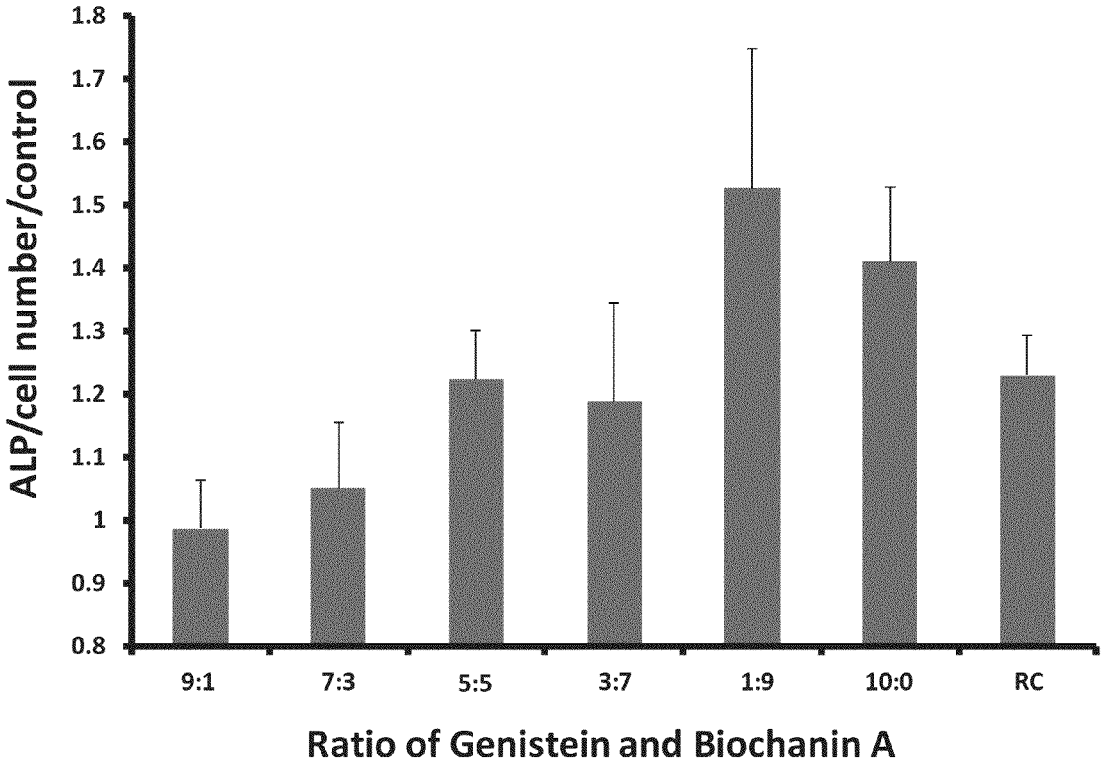

ALP/cell number/control
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
9:1
7:3
5:5
3:7
1:9
10:0
RC
Ratio of Genistein and Biochanin A

Figure 8

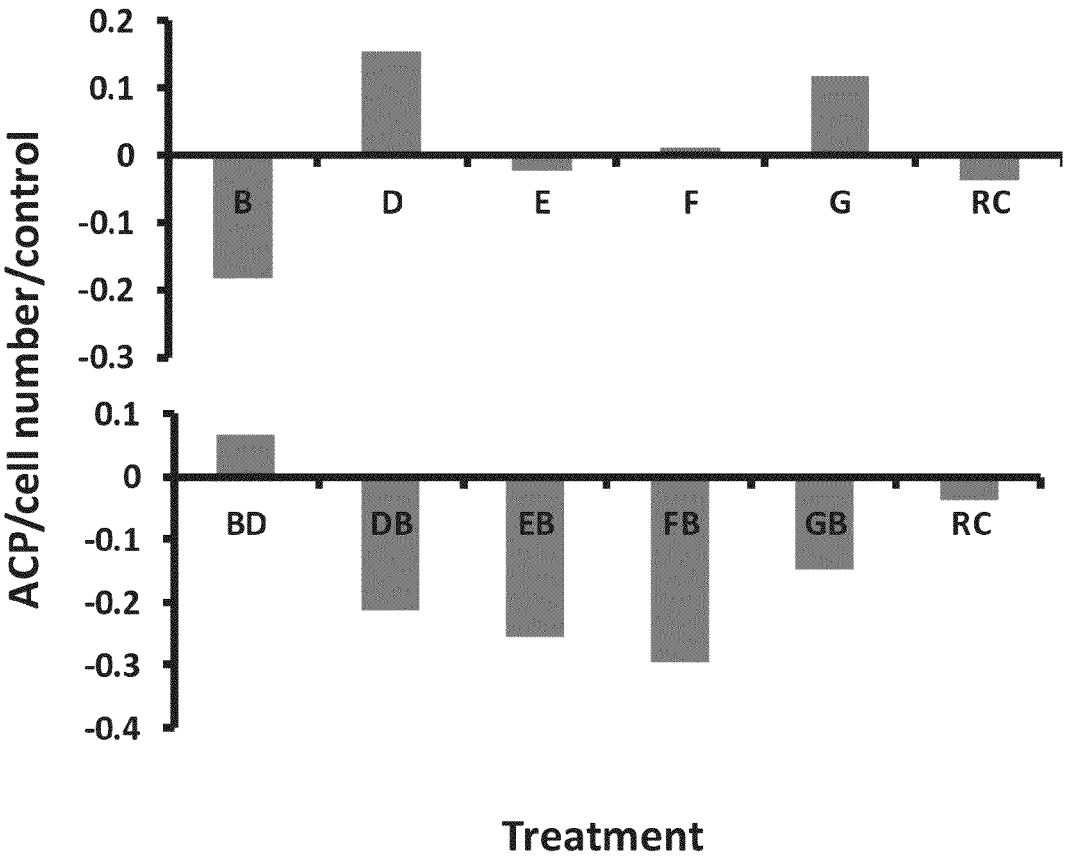

0.2
0.1
0
-0.1
-0.2
-0.3
B
D
E
F
G
RC
0.1
0
-0.1
-0.2
-0.3
-0.4
BD
DB
EB
FB
GB
RC
ACP/cell number/control
Treatment

Figure 9

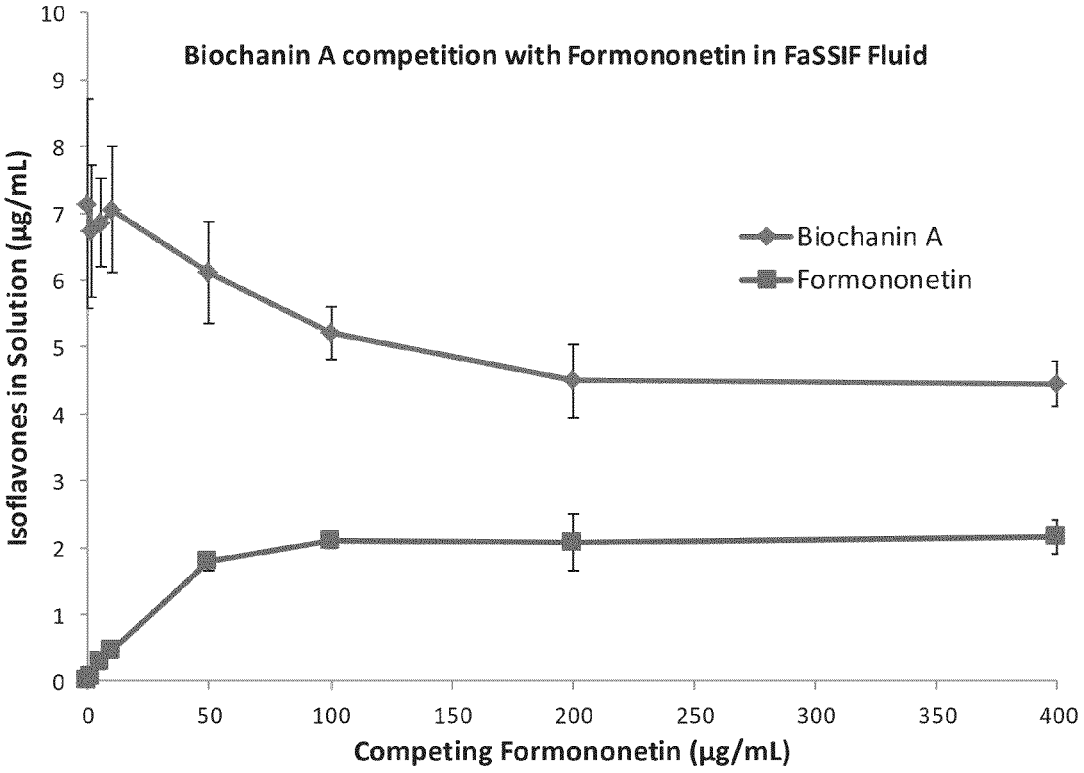

Biochanin A competition with Formononetin in FaSSIF Fluid
Isoflavones in Solution (µg/mL)
Competing Formononetin (µg/mL)
Biochanin A
Formononetin
0
1
2
3
4
5
6
7
8
9
10
0
50
100
150
200
250
300
350
400

Figure 10

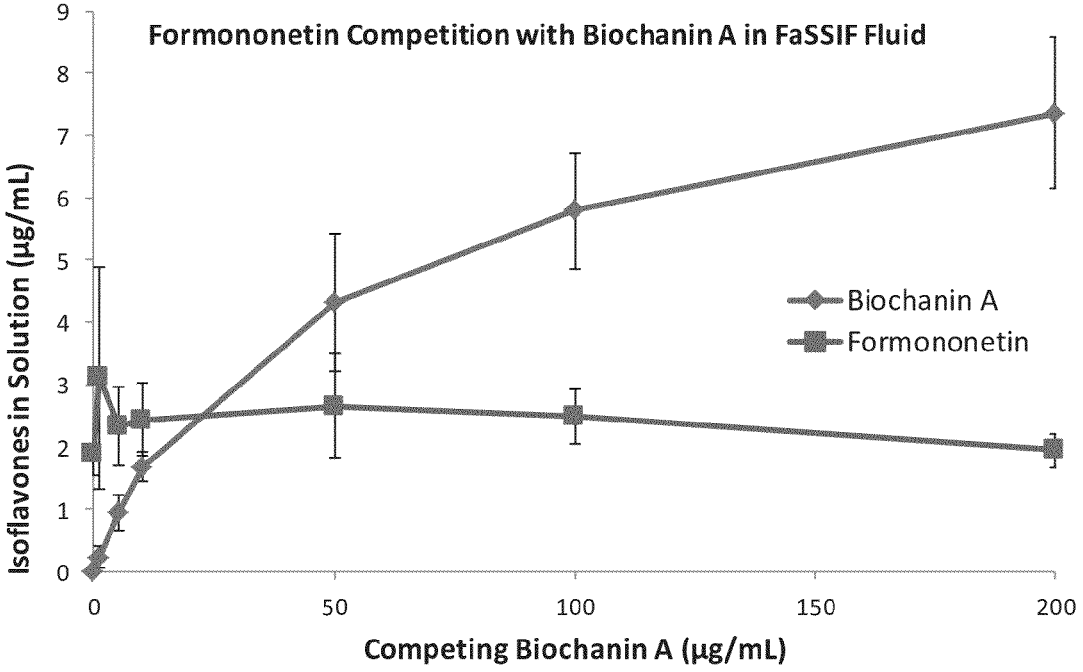

Formononetin Competition with Biochanin A in FaSSIF Fluid
Isoflavones in Solution (µg/mL)
Competing Biochanin A (µg/mL)
Biochanin A
Formononetin
0
1
2
3
4
5
6
7
8
9
0
50
100
150
200

Figure 11

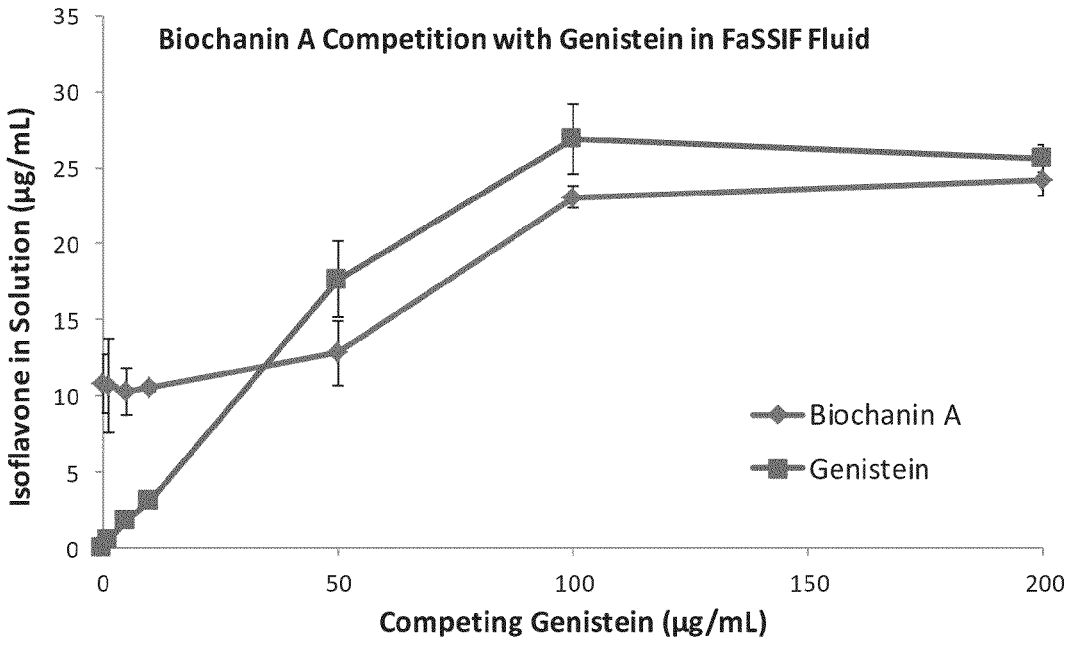

Biochanin A Competition with Genistein in FaSSIF Fluid
Isoflavone in Solution (µg/mL)
35
30
25
20
15
10
5
0
0
50
100
150
200
Competing Genistein (µg/mL)
Biochanin A
Genistein

Figure 12

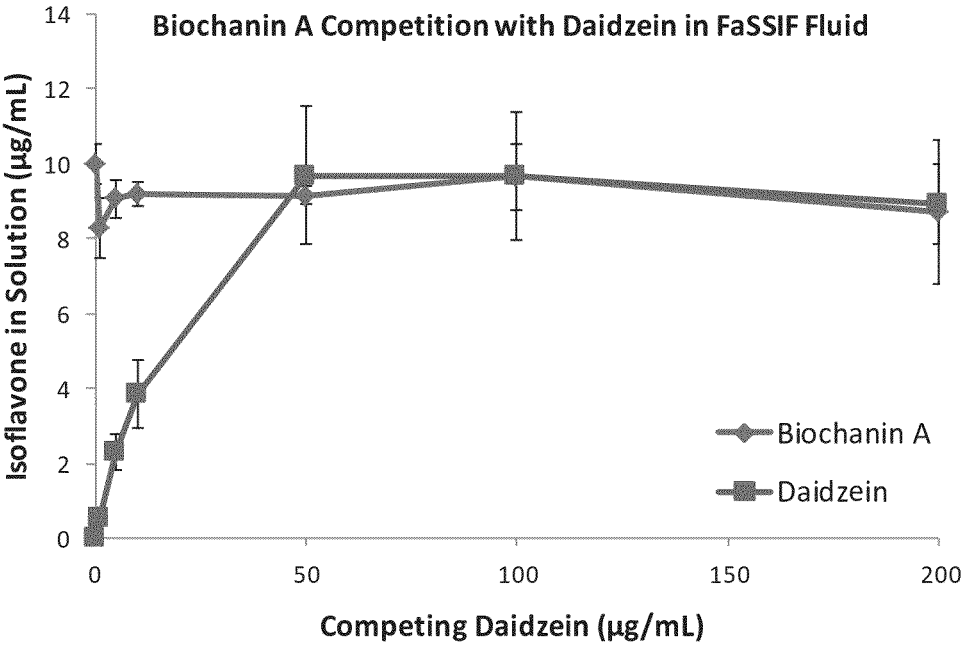

Biochanin A Competition with Daidzein in FaSSIF Fluid
Isoflavone in Solution (µg/mL)
14
12
10
8
6
4
2
0
0
50
100
150
200
Competing Daidzein (µg/mL)
Biochanin A
Daidzein

Figure 13

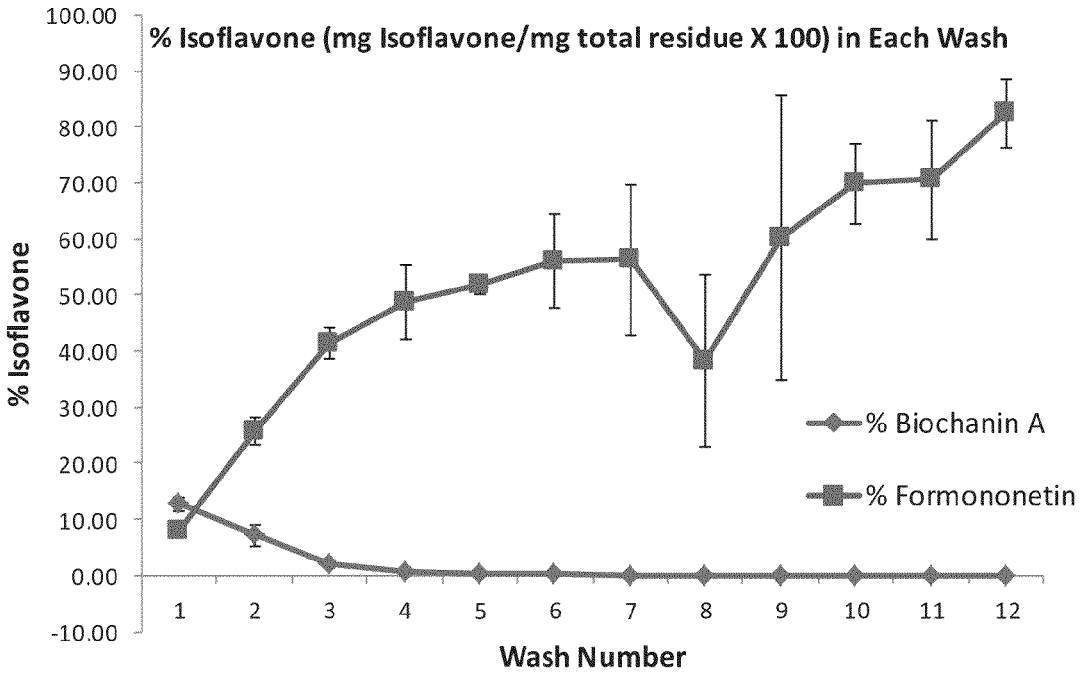

% Isoflavone (mg Isoflavone/mg total residue X 100) in Each Wash
% Isoflavone
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
-10.00
1
2
3
4
5
6
7
8
9
10
11
12
Wash Number
% Biochanin A
% Formononetin

Figure 14

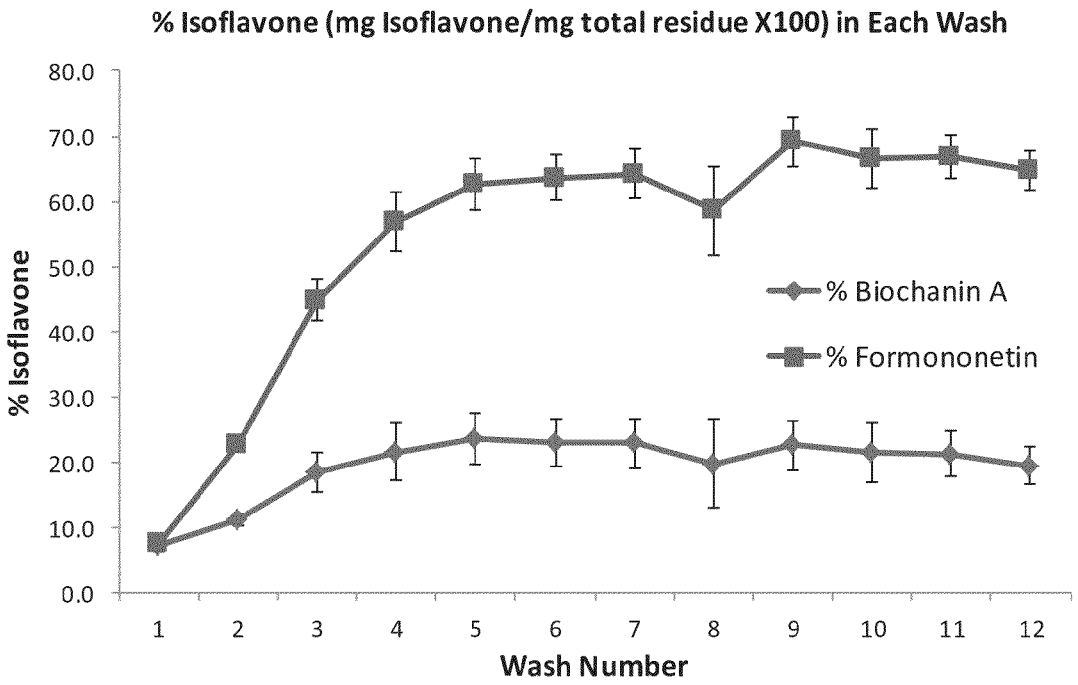
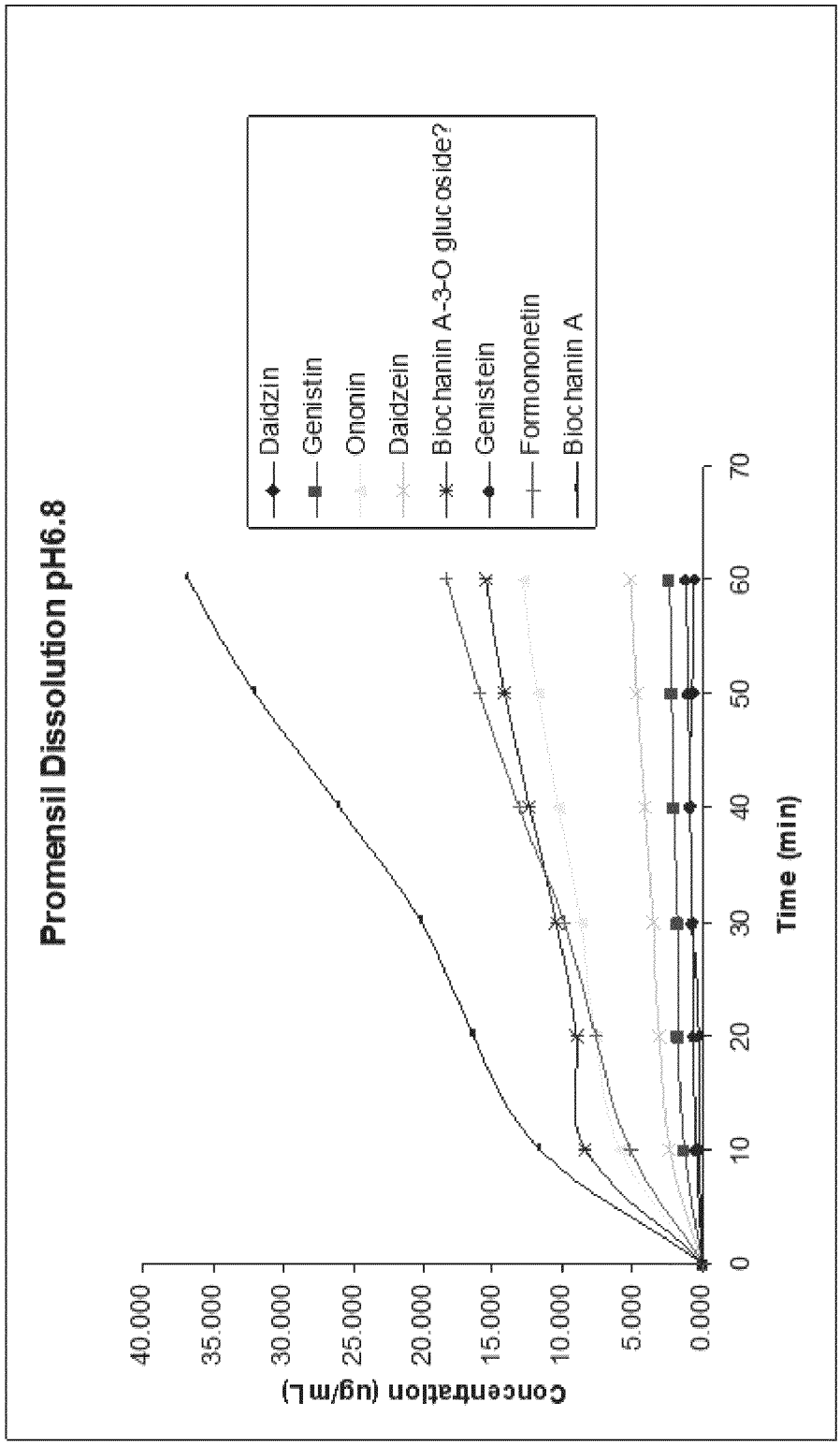

% Isoflavone (mg Isoflavone/mg total residue X100) in Each Wash
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
% Isoflavone
1
2
3
4
5
6
7
8
9
10
11
12
Wash Number
% Biochanin A
% Formononetin

Figure 15


Promensil Dissolution pH6.8
Daidzin
Genistin
Ononin
Daidzein
Biochanin A-3-O glucoside?
Genistein
Formononetin
Biochanin A
Concentration (ug/mL)
40.000
35.000
30.000
25.000
20.000
15.000
10.000
5.000
0.000
0
10
20
30
40
50
60
70
Time (min)

# PHYTOESTROGEN PRODUCT OF RED CLOVER AND PHARMACEUTICAL USES THEREOF

This application is a continuation-in-part application of International Application No. PCT/IB2012/055277, filed Oct. 2, 2012, which claims benefit of U.S. App'l Ser. No. 61/542,253, filed Oct. 2, 2011; this application also is a continuation-in-part application of U.S. application Ser. No. 14/069,740, filed Nov. 1, 2013, which is a continuation of U.S. application Ser. No. 13/251,267, filed Oct. 2, 2011, which is a continuation-in-part of U.S. application Ser. No. 13/028,136, filed Feb. 15, 2011, which claims benefit of U.S. App'l Ser. No. 61/304,589, filed Feb. 15, 2010, the contents of which are incorporated herein by reference in its entirety.

## BACKGROUND OF THE INVENTION

Deficiency of estrogens during menopause can lead to a number of complications including hot flushes, reduced bone density, mood swings, etc. These symptoms are commonly treated with synthetic hormones. Although the rate of bone density reduction can be alleviated, hormone replacement therapy (HRT) was discovered to be associated with increased cardiovascular disorders in one of the largest studies of its kind (Women's health Initiative, WHI) (Seelig, Altura et al. 2004). HRT was also linked to increased risk of breast and ovarian cancer (Fernandez, Gallus et al. 2003, Gambacciani, Monteleone et al. 2003). After the WHI trial results were published, the use of HRT was reduced dramatically. Many postmenopausal women have resorted to alternative therapy because phytoestrogens are generally considered to be safe and efficacious. The use of soy and Red clover (*Trifolium pratense*), which are rich in phytoestrogens, has been on the rise (Beck, Rohr et al. 2005). Despite the trend, clinical trial results on phytoestrogens, however, have been equivocal (Beck, Rohr et al. 2005, Booth, Piersen et al. 2006, Wuttke, Jarry et al. 2007, Ma, Qin et al. 2008). Alternative therapy has not replaced HRT effectively. A recent study showed that the trend of women moving away from HRT has led to an alarming increase in bone fractures and it is estimated that fractures related to menopause is expected to exceed 40,000 per year in women aged 65-69 years (Gambacciani, Ciaponi et al. 2007). Since the side effects of HRT were publicized after the WHI trial, it has since been reevaluated. There is no consensus with regard to HRT's safety among the medical research community. Therefore, a much closer look at the 'less than expected' effects of phytoestrogens should be undertaken because the toxicity profile of this type of products is so much more favorable.

The major bioactive isoflavones in soy are genistein, daidzein, glycitein and prunetin (Setchell and Cassidy 1999). They are also present in their glycoside forms. There are three classes of bioactives in red clover: isoflavones, coumestrols and lignans (Beck, Rohr et al. 2005). The quantity of coumestrols and lignans is small; therefore, their contribution to the overall activity is likely minimal. The major isoflavones in red clover are Biochanin A and Formononetin (Liu, Burdette et al. 2001, Overk, Yao et al. 2005, Booth, Overk et al. 2006). Genistein and daidzein are present in minute quantities. Biochanin A and Formononetin are precursors of their respective active moieties, genistein and daidzein. The conversion takes place in the intestine by intestinal flora and liver, although the relative significance has not been established. Daidzein is converted by bacteria in the colon to form a more estrogenic metabolite, equol. In Red clover, a significant quantity of Biochanin A and Formononetin is in the form of glycosides. The glycosides in soy and red clover are converted to their respective aglycones by the intestinal flora before absorption (Setchell and Cassidy 1999).



Relative absorption of isoflavone glycoside and their respective aglycones is a subject of controversy. Although the cause of controversy is not readily apparent, the low solubility of the aglycones in a preparation may have a profound effect on their dissolution, metabolism and absorption.

Formononetin and Biochanin A are de-methylated by the intestinal micro flora to produce two active metabolites daidzein and genistein, respectively (Hur and Rafii 2000). However, the site of this metabolic pathway is questioned (Tolleson, Doerge et al. 2002).

Metabolism of isoflavones is mainly mediated by Phase II enzymes in the enterocytes and hepatocytes. Although metabolism of individual isoflavones in rats has been well characterized (Jia, Chen et al. 2004, Chen, Lin et al. 2005, Chen, Wang et al. 2005), interaction between components has not been evaluated.

Clinical studies show that extracts of red clover or soy are safe; however, their efficacies are also equivocal (Booth, Piersen et al. 2006). Although there are proprietary products in the market, which have shown potentials for treating or preventing postmenopausal osteoporosis, these products unfortunately, do not have the quality of a drug. The major shortcomings for the design of these products in the market are that they have not taken into consideration of the interplay between pharmacokinetics and pharmacodynamics. In other words, proper dosage and/or dosing interval are empirically decided.

In this invention, the interplay between these "active" components is evaluated and quantified using a proprietary physiologically based pharmacokinetic and pharmacodynamic model (PBPKPD).

The dosages of the new products are a small fraction of those available in the market. The advantage of these products is their consistency. By modifying the mode of delivery, the other advantage of this product is the increase in the bioavailability of the aglycones and eliminates the conversion to their respective bioactive metabolites in the colon, which leads to variability in efficacy.

## SUMMARY OF THE INVENTION

The present invention discloses a composition of active ingredients in Red clover, which are optimized to reduce the rate of bone loss in postmenopausal women by enhancing bone remodeling. In one embodiment, the composition comprises at least 80% of Biochanin A, and no more than 20% of genistein. In another embodiment, the composition comprises at least 80% of Biochanin A, and at least 2% of genistein. In another embodiment, the composition further comprises Formononetin, daidzein, or a combination of Formononetin and daidzein. In another embodiment, the composition is formulated as a parenteral, buccal, sublingual, and other non-oral dosage forms including, but not limited to, topical, subcutaneous, intramuscular and intravenous dosage forms.

## DETAILED DESCRIPTION OF THE FIGURES

FIG. **1** shows a typical LC/MS chromatogram showing the composition of a Red clover extract.

FIG. **2** shows the metabolism of the isoflavone mixtures by human fecal bacteria.

FIG. **3** shows the metabolism of the isoflavone mixtures by human intestinal microsomes.

FIG. **4** shows the metabolism of the isoflavone mixtures by human hepatocytes.

FIG. **5** shows the effects of individual Red clover isoflavones on osteoblast differentiation of MC3T3 cells. The total concentration of isoflavone in each treatment is 10 μM. B: Biochanin A; D: daidzein; E: equol; F: formononetin; G: genistein; and RC: Red clover extract. ALP/cell number ratio obtained in each treatment is normalized by the control to quantify relative osteoblast activities.

FIG. **6** shows the effects of Red clover isoflavone mixtures on osteoblast differentiation of MC3T3 cells. The total concentration of isoflavone in each treatment is 10 μM. For pair treatment, the ratio is 1:9. B is Biochanin A; DB is 1 μM of daidzein and 9 μM of Biochanin A; EB is 1 μM of equol and 9 μM of Biochanin A; FB is 1 μM of Formononetin and 9 μM of Biochanin A; GB is 1 μM of genistein and 9 μM of Biochanin A; and RC is Red clover extract. ALP/cell number ratio obtained in each treatment is normalized by the control to quantify relative osteoblast activities.

FIG. **7** shows the effects of progressive increase of genistein in a mixture of genistein and Biochanin A on the osteoblast differentiation of MC3T3 cells. The total concentration of isoflavone in each treatment is 10 μM. Effect of Red clover extract is also tested. ALP/cell number ratio obtained in each treatment is normalized by the control to quantify relative osteoblast activities.

FIG. **8** shows the inhibition of osteoclast differentiation by Red clover isoflavones. The total concentration of isoflavone in each treatment is 10 μM. B: Biochanin A; D: daidzein; E: equol; F: formononetin; G: genistein; and RC: Red clover extract. For pair treatment, the ratio is 1:9. BD is 1 μM of Biochanin A and 9 μM of daidzein; DB is 1 μM of daidzein and 9 μM of Biochanin A; EB is 1 μM of equol and 9 μM of Biochanin A; FB is 1 μM of Formononetin and 9 μM of Biochanin A; GB is 1 μM of genistein and 9 μM of Biochanin A; and RC is Red clover extract. ACP/cell number ratio obtained in each treatment is normalized by the control to quantify relative osteoclast activities.

FIG. **9** shows the effects of Formononetin on the solubility of Biochanin A in fasted simulated intestinal fluid (FaSSIF). The amount of Biochanin A in the mixture is 200 μg/mL.

FIG. **10** shows the effects of Biochanin A on the solubility of Formononetin in fasted simulated intestinal fluid (FaSSIF). The amount of Formononetin in the mixture is 50 μg/mL.

FIG. **11** shows the effects of genistein on the solubility of Biochanin A in fasted simulated intestinal fluid (FaSSIF). The amount of Biochanin A in the mixture is 200 μg/mL.

FIG. **12** shows the effects of daidzein on the solubility of Biochanin A in fasted simulated intestinal fluid (FaSSIF). The amount of Biochanin A in the mixture is 200 μg/mL.

FIG. **13** shows the Isoflavone profiles of a commercial Red clover extract (Shaanxi, 40% total phytoestrogen) after sequential extraction with methanol.

FIG. **14** shows the Isoflavone profiles of a commercial Red clover extract (Acetar, 40% total phytoestrogen) after sequential extraction with methanol.

FIG. **15** shows a dissolution profile of two PROMENSIL tablets, each contains 40 mg of total phytoestrogens. The content of Biochanin A and Formononetin was not completely released.

## DETAILED DESCRIPTION OF THE INVENTION

Biochanin A and Formononetin are the major components in Red clover. Their colonic metabolites, genistein and daidzein are also present in minute quantities (Beck, Rohr et al. 2005). A colonic metabolite of daidzein, equol, has been shown to have the highest estrogenicity among the red clover phytoestrogens (Magee 2011).

As shown below, the two major aglycones of Red clover, Biochanin A and Formononetin are highly insoluble in the gastrointestinal fluids. Daidzein and genistein, two minute components in Red clover, are also insoluble, although they are more soluble than Biochanin A and formononetin.

In the present invention, Biochanin A, formononetin, daidzein and genistein are found interacting with each other at the solubility level. The presence of one phytoestrogen may enhance or inhibit the solubility of the other phytoestrogen.

Lack of solubility, high first-pass gut and liver metabolism and colonic bacteria metabolism are responsible for the highly variable and extremely low bioavailability of the active components.

In one embodiment, the present invention provides a composition comprising active ingredients in Red clover, which are optimized to reduce the rate of bone loss in postmenopausal women by modulating bone remodeling. In one embodiment, the composition comprises at least 80% of Biochanin A, and no more than 20% of genistein. In another embodiment, the composition comprises at least 80% of Biochanin A, and at least 2% of genistein. In another embodiment, the ratio of Biochanin A and genistein ranges from 8:1 to 20:1.

In one embodiment, the composition contains at least 80% of Biochanin A, no more than 8% of genistein, and no more than 6% each of formononetin and daidzein. In one embodiment, the composition contains at least 80% of Biochanin A, at least 5% of genistein, and no more than 6% each of formononetin and daidzein. In one embodiment, the ratio of Biochanin A and Formononetin ranges from 20:1 to 10:1. In another embodiment, the ratio of Biochanin A and daidzein ranges from 20:1 to 10:1.

In one embodiment, the composition comprises a dosage of total isoflavones ranging from 1 to 10 mg.

In one embodiment, the compositions disclosed herein are obtained through synthetic processes, synthetic sources or natural sources.

In one embodiment, the present invention provides dosage forms of the compositions that will minimize first-pass metabolism, enhance exposure to active ingredients and minimize inter-individual variability.

In one embodiment, the composition is formulated as parenteral, buccal, sublingual, and other non-oral dosage forms including, but not limited to, topical, subcutaneous, intramuscular and intravenous dosage forms.

In one embodiment, the composition is formulated in the form of tablets, granules, injection, powder, solution, suspension, or capsules.

In one embodiment, the present invention also provides methods of using the compositions disclosed herein for modulating bone remodeling, comprising the step of administering the composition to a subject in need thereof. In one embodiment, the composition is formulated as parenteral, buccal, sublingual, or other non-oral dosage forms including, but not limited to, topical, subcutaneous, intramuscular and intravenous dosage forms.

In one embodiment, the present invention also provides methods of using the compositions disclosed herein for treating or preventing osteoporosis, comprising the step of administering the composition to a subject in need thereof. In one embodiment, the composition is formulated as parenteral, buccal, sublingual, or other non-oral dosage forms including, but not limited to, topical, subcutaneous, intramuscular and intravenous dosage forms.

The invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative, and are not meant to limit the invention as described herein, which is defined by the claims which follow thereafter.

Throughout this application, various references or publications are cited. Disclosures of these references or publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains. It is to be noted that the transitional term "comprising", which is synonymous with "including", "containing" or "characterized by", is inclusive or open-ended and does not exclude additional, un-recited elements or method steps.

## Example 1

The objective of this study is to track the events that occur in the lumen of the gastrointestinal tract. The goals are to identify the stability of Red clover components, their physical and enzymatic stability, solubility and absorbability.

Twenty-five red clover extracts containing a diverse composition of Biochanin A, Formononetin, Genistein, Daidzein and their glucosides, along with other minute quantities of coumestrols and lignans have been prepared either using solvent extraction or a variety of cultivars. In one embodiment, the aerial portion of red clovers, leaves, stems or leaves and stems, were dried powdered. The plant material was extracted with 50% ethanol at 50° C. for 1 hour. The resultant sample was centrifuged and the ethanolic component was removed and dried.

A chromatographic analysis showed that the major ingredients in these extracts are the glucosides of Formononetin and Biochanin A and their respective aglycones (FIG. **1**). Tiny amounts of genistein, daidzein and their glycosides were also found. These data are consistent with what is reported in the literature (Krenn, Unterrieder et al. 2002).

A study of the stability of the key components of a Red clover extract in artificial gastric and intestinal juice showed that the glucosides were partially (<25%) converted to their respective aglycones.

According to the literature, Formononetin and Biochanin A are de-methylated by the intestinal micro flora to produce two active metabolites daidzein and genistein, respectively (Hur and Rafii 2000). However, the importance of this metabolic pathway at this site is questioned (Tolleson, Doerge et al. 2002). To understand the relative importance of fecal metabolism, the metabolic rate of red clover phytoestrogens was measured.

Fresh human fecal samples were collected from 4 volunteers. Five grams of each were pooled together and mixed well with 30 mL BHI culture medium. The fecal suspension was centrifuged at 200 g for 5 min and supernatant was decanted and centrifuged at 5,000 g for another 30 min. The resultant precipitate was re-suspended with 10 mL BHI medium to produce intestinal micro flora solution.

As the biotransformation of drugs by human intestinal bacteria was determined in a 5 mL incubation system containing 250 μL, intestinal microflora solution, 50 μL, stock solution in DMSO in BHI medium. The incubation system was anaerobically incubated at 37° C. in a GasPak™ EZ Anaerobe Pouch System for 0 h, h, 72 h, and 120 h for red clover isoflavones (the final concentrations for Biochanin A, daidzein, equol, Formononetin, and genistein were 100 μM each). Zero-minute incubations served as controls. Reactions were stopped by extracting samples with ml of ethanol twice. The two ethanol extractions were combined, dried and re-suspended in 80% methanol for HPLC/MS analysis.

Red clover isoflavones are shown to be metabolized extensively by human intestinal microflora (FIG. **2**). When Biochanin A was incubated with intestinal microflora, dihydrobiochanin A, genistein, and dihydrogenistein were formed. Daidzein was metabolized into dihydrodaidzein, and equol. Equol was the most resistant to bio-transformation. At the end of 120 hours, there was still over 60% of equol left in the incubation media, while there were less than 5% of Biochanin A, daidzein, and genistein left. The bio-transformed products of equol were not identified in this study. Formononetin was biotransformed into dihydroformononetin, dihydrodaidzein, daidzein, and equol. At the end of 120-hour incubation, there was over 20% of Formononetin remained. Genistein was bio-transformed into dihydrogenistein.

This set of studies clearly showed that extensive Phase I metabolism occurs in the lower part of the intestinal lumen.

Red clover extracts were subjected to permeability measurements using Caco-2 and MDCK cells. Permeability across these barriers provides an indication of absorbability.

The permeability values of Formononetin, Biochanin A, daidzein and genistein are quite high, suggesting that these components are highly absorbable (Table 1). Equol has also been shown to be absorbable. However, the glucosides of the aglycones such as Biochanin A glucoside and ononin have poor permeability suggesting the bioavailability of the sugar conjugates are poorly absorbed. These results are consistent with that reported in the literature in that when these glycosides are administered to either animals or humans, no glycosides could be detected in the blood stream (Setchell, Brown et al. 2002).

TABLE 1

CaCo-2 permeability of isoflavone in a Red clover extract

| Isoflavones | Mean Peff, cm/sec | STDEV |
|---|---|---|
| Biochanin A glucoside | 1.64E−08 | 1.11E−09 |
| Biochanin A | 1.08E−05 | 3.83E−07 |
| Daidzein | 2.66E−05 | 1.11E−06 |
| Daidzin | 5.36E−07 | 8.30E−08 |
| Formononetin | 2.20E−05 | 6.85E−07 |
| Genistein | 2.75E−05 | 1.22E−06 |
| Genistin | 3.46E−07 | 9.20E−08 |
| Ononin | 1.22E−07 | 1.93E−08 |

The results from the permeability study show that it would be beneficial to convert all the glucosides to their respective aglycones. Two advantages of adopting this strategy: a. the variability in the rate and extent of conversion from glucosides to aglycones between subjects will be removed. A more consistent pattern of aglycone absorption is anticipated. b. dosage calculation for the bioactives will be reduced to the aglycones only. This simplifies the standardization process.

An optimal extract of Red clover should consist of the aglycones only. An enzymatic or chemical conversion of the glucosides to their respective aglycones prior to extraction will be desirable. This can be accomplished using the literature methods (Tsao, Papadopoulos et al. 2006).

## Example 2

The objectives of this example are to evaluate gut and liver metabolism of Biochanin A, Formononetin, daidzein, genistein and equol. Parameters obtained from these studies are used for estimating the pharmacokinetics of these five components.

Human liver microsomes, intestinal microsomes, and hepatocytes of human female origin were purchased from XenoTech. All chemicals were purchased from Sigma-Aldrich. Isoflavones (biochanin A, daidzein, equol, Formononetin, and genistein) were first dissolved in DMSO and then mixed according to a randomized table, consisting of 60 samples. The final DMSO in buffer or media was kept at 0.1%. Protocols supplied by XenoTech Inc., the supplier, were used for glucuronidation with microsomal incubation, and hepatocyte incubation. Samples were analyzed using LC/MS.

FIG. **3** shows that metabolism of the mixtures by human intestinal microsomes: Biochanin A (5.41E-4 ml/min/mg protein)>genistein (4.28E-4 ml/min/mg protein)>equol (1.07E-ml/min/mg protein)>daidzein (6.32E-5 ml/min/mg protein)>Formononetin (7.31E-5 ml/min/mg protein).

FIG. **4** shows the rate of metabolism of the mixtures by human hepatocytes. The rates are: equol (1.21E-5 ml/min/million cells)>biochanin A (8.88E-6 ml/min/million cells) >genistein (5.14E-6 ml/min/million cells)>daidzein (4.07E-6 ml/min/million cells)>Formononetin (3.45E-6 ml/min/million cells).

From these studies, it is clearly shown that there are no metabolic interactions between the five components. In these metabolic studies, no Phase I metabolites were detected suggesting that the formation of Phase I metabolites, such as daidzein and genistein are formed in the intestinal lumen (Example 1). This piece of information is important in that the rate of formation of these metabolites is dependent on the solubility of Formononetin and Biochanin A. These results are consistent with that reported by Howes et al (2002) in that the peak time of the Phase I metabolites is delayed.

## Example 3

The objectives of this study are to evaluate the effects of individual isoflavones of Red clover and their combinations on osteoblast and osteoclast differentiation.

Materials and Methods

Effects of isoflavones on the differentiation of osteoblast in MC3T3 cells and differentiation of osteoclast in Raw264.7 cells were determined as described in Ge et al., 2006 and followed Garcia Palacios et al., 2005. Cell numbers were measured with CellTag from Li-Cor Biosciences. Both activities of alkaline and acid phosphatase were measured with a plate reader at 405 nm.

Isoflavones were first dissolved in DMSO and stock solutions were prepared at 10 mM and the final concentration of total isoflavones in test solution was 10 μM.

Osteoblast and osteoclast differentiations were quantified by measuring activities of alkaline phosphatase (ALP) and acidic phosphatase (ACP). ALP is highly expressed by the mature osteoblasts and ACP is expressed by osteoclasts. Values of integrated intensity of fluorescence from Celltag staining serve as a correction factor for the difference in cell numbers. Therefore, ALP/cell number and ACP/cell number ratios are used to quantify osteoblast and osteoclast activities.

Results

Osteoblast Differentiation

Confluence MC3T3 cells were treated with 10 μM of isoflavones for 1 week and then the activity of alkaline phosphatase (ALP) was measured as an indicator of differentiation. Although the difference among isoflavone treatments was not significant, cells treated with Biochanin A consistently showed the highest ALP activity (FIG. **5**). To examine if there were any synergistic effects, two isoflavones were mixed in a 1:9 ratio and tested in the final concentration of 10 μM. Mixtures with higher ratios (90%) of biochanin A were usually more effective in enhancing the osteoblast differentiation of MC3T3 cells than individual isoflavones alone or their combinations. In the example shown here cells treated with daidzein:Biochanin A (1:9) and genistein:Biochanin A (1:9) had higher ALP activities than Biochanin A alone (FIG. **6**). To examine the effect of genisterin:Biochanin ratio on osteoblast differentiation, genistein was mixed with an increased concentration of Biochanin A (an increment of 10%). With the increased concentration of Biochanin A, the differentiation enhancing ability of the mixture increased and then dropped off when the mixture only contained Biochanin A (FIG. **7**).

Osteoclast Differentiation

Raw246.7 cells were treated with MCSF and RANKL to stimulate the differentiation of osteoclasts. Isoflavones were added at the final concentration of 10 μM to examine their ability to inhibit differentiation. Cells treated with Biochanin A and its mixtures showed the highest inhibition in osteoclast differentiation (FIG. **8**).

Conclusions

Contrary to its low estrogenicity (Beck, Unterrieder et al. 2003), Biochanin A is found to be most effective in enhancing the differentiation of osteoblast and to inhibit the differentiation of osteoclasts. Mixtures of Red clover aglycones containing high proportions of Biochanin A show synergistic effects. In one embodiment, the preferred ratios of the components at the site of action are 80 to 90% of Biochanin A, up to 20% genistein, and no more than 10% each of daidzein and Formononetin.

## Example 4

The objectives of this study are: 1. To evaluate solubility interactions among the four phytoestrogens, which are native to Red clover, namely, Biochanin A, Formononetin, daidzein and genistein; 2. To evaluate potential differences in physicochemical properties of extracts containing the same amounts of phytoestrogens.

Materials and Methods

Interactions Among Isoflavones

Isoflavones: daidzein, genistein, Formononetin and Biochanin A were obtained from Indofine. Simulated intestinal fluid buffer powder mimicking a fasted state (FaSSIF) was obtained from Biorelevant SIF media, Biorelevant.com, Switzerland.

To prepare accurate concentrations of isoflavones in microtubes, stock solutions of individual isoflavones were prepared at 1 mg/mL in methanol. The amount of isoflavone designated to be held constant was provided at a concentration that well exceeded (about 20×) the saturation concentration for that isoflavone. The appropriate amount of stock was transferred to each microtube and the material was dried down in a vacuum centrifuge.

In the case of Biochanin A competition with other flavones (daidzein, genistein and formononetin), the amount of Biochanin A was held constant at 200 μg in 1 ml buffer (saturation concentration for Biochanin A in FaSSIF is about 8 μg/mL). The competing isoflavone was prepared at 0, 1, 5, 10, 50, 100, 200 μg/mL (FIGS. **11** and **12**) and, in the case of Formononetin, 400 μg/mL (FIG. **9**).

In the case of Formononetin competition with Biochanin A, the amount of Formononetin was held constant at 50 μg in 1 ml buffer (saturation concentration for formononetin in FaSSIF is about 2 μg/mL). The competing Biochanin A was prepared at 0, 1, 5, 10, 50, 100 and 200 μg/mL (FIG. **10**).

Each tube was then reconstituted with 1 mL of FaSSIF buffer, sonicated and allowed to equilibrate with occasional agitation for 24 hours at 37° C. This produced a solution that contained saturated isoflavone concentrations mimicking mammalian intestinal conditions.

At the end of 24 hours each tube containing isoflavones and FaSSIF buffer was briefly centrifuged at 5000 rpm in a microcentrifuge held at 37° C. (2 minutes). A portion of the supernatant (400 μL) was then immediately placed in a centrifugal filter unit (UltraFree-MC-GV 0.22 μM) and the sample filtered by centrifugation (8000 rpm, 5 minutes, 37° C.). Upon filtration 200 μL filtrate was immediately placed in a microtube and 200 μL, methanol added to ensure that the isoflavones remained in solution. The sample was mixed and 200 μL, of the mixture was transferred to injection vials provided with 200 μL, polypropylene injection inserts.

The samples were analyzed by HPLC with diode array detection at 260 nm using 20 μL, injections.

Results

When Biochanin A was placed in media representing fasted digestive juice at a concentration of 200 μg/mL at 37° C. (an amount about 25 times the soluble saturation value), the amount in solution was determined to be about 7.1 μg/mL (FIG. **9**). As Formononetin was introduced, Biochanin A saturation concentration dropped in a dose dependent manner and was reduced to about 4.5 μg/mL in the presence of 400 μg/mL formononetin (FIG. **9**).

When the experiment was done holding formononetin at 50 μg/mL (about 25 times Formononetin's saturation solubility in fasted media), increasing concentrations of Biochanin A did not affect the saturation concentration of Formononetin (FIG. **10**). It is concluded that the presence of Biochanin A does not influence the saturation concentration of the much less soluble Formononetin.

In experiments where Biochanin A solubility was investigated in the presence of varying concentrations of genistein, a different set of results was obtained. When Biochanin A was placed in media representing fasted digestive juice at a concentration of 200 μg/mL at 37° C. the amount in solution was determined to be about 10.8 μg/mL (FIG. **11**). As genistein was introduced, Biochanin A saturation concentration increased in a dose dependent manner and reached about 24.2 μg/mL in the presence of 200 μg/mL genistein (FIG. **11**). It is concluded that the solubility of Biochanin A is enhanced by the presence of genistein in fasted digestive medium.

In experiments where Biochanin A saturation solubility was investigated in the presence of varying concentrations of daidzein, a set of results different from both the Formononetin and genistein experiments were obtained. When Biochanin A was placed in media representing fasted digestive juice at a concentration of 200 μg/mL at 37° C. the amount in solution was determined to be about 10.0 μg/mL (FIG. **12**). As daidzein was introduced, the Biochanin A saturation concentration remained unaffected and was about 8.7 μg/mL in the presence of 200 μg/mL daidzein (FIG. **12**). It is concluded that the solubility of biochanin A is unaffected by the presence of daidzein in fasted digestive media.

This set of studies clearly showed that interactions among isoflavones are not predictable. The solubility of Biochanin A in simulated intestine juice is reduced by Formononetin, enhanced by genistein, and not affected by daidzein.

Similar results are obtained when simulated intestinal juice mimicking the fed state was used (data not shown).

Solubility of Isoflavones in Red Clover Extracts

Two Red clover extracts containing 40% total isoflavones were examined: Shaanix Tianzun BN 078201205123 and Acetar TYR081023.

One gram extract was placed in a disposable 12 mL glass screw top test tube. 10 mL of 100% methanol was added and the tube was capped. The tube was mixed and placed in an ultrasonic water bath for 5 minutes. The tube was then shaken every 15 minutes for 1 hour. Mixing was done at room temperature. At the end of the 1 hour incubation the tube was centrifuged (Eppendorf 5804 R, 1500 rpm, 10 minutes) and the supernatant was collected and set aside. Another 10 mL of methanol was introduced to the tube on top of the sediment and the material was sonicated, mixed and incubated as described above. This process was repeated such that 12 washes from the material were collected. The precipitate from the final wash was re-suspended in methanol.

A 100 μL aliquot collected from each 10 mL wash was diluted 1:10 with 80% methanol, centrifuged and the supernatant analyzed by HPLC with diode array detection at 260 nm using 5 or 20 μL injections. 20 μL injections were used for later washes (wash 6-12) in which daidzein, genistein and Biochanin A concentrations were much lowered.

The remaining wash supernatants as well as the re-suspended final residue were individually dried down in preweighed microtubes to provide an estimate of solid weight recovered in each wash.

An estimate of isoflavone concentrations in the original extracts was made by dissolving the extracts at 1 mg/mL in 80% methanol with warming and sonication. An aliquot was diluted 1:10 with 80% methanol and centrifuged. A 5 μL injection was analyzed by HPLC.

The two 40% isoflavone products extract (Acetar and Shaanxi) are found to be different from each other. There was a difference in appearance between the two 40% extracts as one has a color of dark green-gray (Acetar) and the other one is off white (Shaanxi) after extraction with methanol.

The Acetar extract did not release isoflavones as rapidly when compared to that of Shaanix (Comparing FIGS. **13** and **14**). Biochanin A was still being extracted after 12 sequential extracts. It appears that something in the Acetar extract is binding the isoflavones and only slowly releasing them into the methanol (FIG. **14**).

The results of this study clearly showed that isoflavones from different Red clover extracts produced using different procedures could have vastly different solubility. Since absorption of isoflavones is highly dependent on their solubility, isoflavones prepared from sources or materials with identical labels may have different bioavailability. This may in part explain the inconsistent clinical results reported in the literature (Booth, Piersen et al. 2006).

The hypothesis that solubility may be an issue of phytoestrogen absorption was tested by examining the dissolution profile of a commercially available Red clover product, PROMENSIL® (30 tablets in a box, Lot # [B] 48449, Exp. 03/2011).

FIG. **15** shows that the dissolution of phytoestrogens in the product is not complete, lending evidence to support the idea that an inappropriately formulated product will perform erratically because of absorption issues. It should also be pointed out that the phytoestrogens in PROMENSIL® consist of both aglycones and their glucosides. Compounding the bioavailability issue, both of these species are not completely dissolved under the experimental condition studied.

Example 5

The protocol used by (Moon, Sagawa et al. 2006) was used for measuring plasma protein binding of the absorbable aglycones. Parameters have been used for PBPK simulation. Conjugates of Biochanin A, Formononetin, Genistein, Daidzein

and Equol are predominant components in plasma. The respective aglycones constitute less than 5% of the total concentration. Plasma protein binding of biochanin A, Formononetin, genistein and equol are over 97% and daidzein was approximately 80%.

Example 6

The objective of this example is to employ the proprietary pharmacodynamic/pharmacokinetic (PBPK) model to simulate the pharmacokinetic behavior of the active phytoestrogens in Red clover.

Results from Examples 1, 2 and 4 are used as inputs into the proprietary PBPK model to simulate plasma concentration profiles of the four phytoestrogens: Biochanin A, Formononetin, daidzein and genistein and their Phase II metabolites.

Using the parameters generated in Examples 1, 2 and 4, the proprietary PBPK model was adapted to describe the pharmacokinetics of Red clover isoflavones. The model was considered validated when the simulated results of Area Under the Curve (AUC) values of the Phase II metabolites are agreeable (within 2-fold, Table 2) with that published by Howes et al. (2002). Plasma levels of Phase II metabolites of the four isoflavones as reported by Howes et al. (2002) and as simulated using the proprietary PBPK model is compared in Table 2 (second and third columns, Table 2). The two sets of values are found to be agreeable, indicating the simulated results obtained are valid.

TABLE 2

Comparison of plasma levels of isoflavones obtained from clinical samples and simulation results using the PBPK model

| | $AUC_{0-24}$, ng*h/mL | | | |
|---|---|---|---|---|
| | Phase II metabolites | | Aglycone (simulation) | |
| | Howes' data* | Simulation | Oral | IV |
| Formononetin | 112 ± 35 | 123 | 9.47 | 1989 |
| Biochanin A | 518 ± 518 | 519 | 4.99 | 2422 |
| Daidzein | 891 ± 135 | 693 | 7.49 | 261 |
| Genistein | 1463 ± 115 | 1231 | 3.85 | 229 |

*Data reported by Howes et al (2002). Two Promensil tablets containing 32 mg of Formononetin, 49 mg of Biochanin A, 3 mg of daidzein and 3 mg of genistein were administered to subject per day.

The PBPK model was then used to simulate the AUC values of isoflavones after oral and intravenous administration. Plasma levels of aglycone of the four isoflavones after oral and intravenous administration (IV) of identical dose of isoflavones as that of Promensil are simulated using the PBPK model (fourth and fifth columns, Table 2). Comparing the AUC values obtained, it is observed that the AUC values of aglycone obtained after intravenous administration is approximately 20 to 500 times higher than those after oral administration.

This simulation suggests that bioavailability of phytoestrogens would be enhanced by administering the compounds via non-oral route such as intravenous route.

Howes et al's (2002) data also showed that AUC values among subjects are highly variable (>10 fold). This variation can be explained by the instability of glucosides, low solubility of the aglycones, interaction among aglycones at the solubility level, aglycone metabolism of the aglycones by colonic bacteria and high first-pass gut and liver metabolism. The end result is low bioavailability (0.2 to 3%).

High variability, low bioavailability and solubility limited absorption are the major causes for therapeutic failure. In a recent review (Lagari and Levis 2014), it was reported that there was a much higher proportion of clinical trials showing Red clover phytoestrogens were ineffective in treating postmenopausal bone loss and climacteric symptoms than the effective ones. Dosages used for clinical trials went as high as 80 mg total phytoestrogens.

Based on the results described in this invention, failure of Red clover therapy is not surprising because low solubility, high first-pass effects and variable colonic metabolism are key factors which lead to low bioavailability and high inter-individual variability of the phytoestrogens.

Limited solubility of phytoestrogens in the small intestine may be responsible for the lack of a dose-dependent increase in clinical response to Red clover isoflavones (Booth, Piersen et al. 2006).

Commercially, it is common to find Red clover extracts containing higher proportions of Formononetin. As demonstrated in the present invention, Biochanin A is found to be the most effective and Formononetin is shown to lower Biochanin A's solubility in simulated intestinal fluid. Thus Red clover extracts containing high Formononetin are ineffective.

In the realm of solubility limitations, an effective combination of Red clover phytoestrogens should contain very low levels of Formononetin (<2 to 10%).

Genistein has been found to have dual functions. It enhances the solubility of Biochanin A and acts synergistically with Biochanin A in enhancing bone remodeling.

A low percentage of daidzein and Formononetin has also been found to have synergistic antiresorptive effects.

Taking together, an ideal combination of Red clover phytoestrogens should contain a high content of Biochanin A (>80%) and smaller contents of genistein, daidzein and Formononetin.

To avoid extensive first-pass effects, solubility issues and variable colonic metabolism, an alternative route of administration other than oral should be employed. Dosage forms for parenteral, topical, subcutaneous, intramuscular, intravenous, buccal or sublingual administration should be employed.

In one embodiment, the dosage of phytoestrogens can be less than 5-10% of the normal clinical dose, which is 80 mg. These low dosages should provide significantly higher plasma levels of Biochanin A, genistein, daidzein and Formononetin when compared to a regular 80 mg dose of Red clover isoflavones.

Assuming an 80 mg dose is marginally active (Lagari and Levis 2014), the combination of phytoestrogens as disclosed herein would greatly enhance clinical effectiveness of Red clover phytoestrogens.

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What is claimed is:

**1**. An in vitro method of enhancing osteoblast differentiation, comprising the step of contacting osteoblasts with a composition comprising biochanin A and genistein, wherein the ratio of biochanin A to genistein is 9:1, and the composition comprises a dosage of total phytoestrogens ranging from 1 to 10 mg, wherein the concentration of biochanin A is 9 μM and the concentration of genistein is 1 μM.

**2**. The method of claim **1**, wherein the composition comprises the dosage of total phytoestrogens no more than 10 mg.

**3**. The method of claim **1**, wherein said composition inhibits osteoclast differentiation.

**4**. An in vitro method of inhibiting osteoclast differentiation, comprising the step of contacting osteoclasts with a composition comprising biochanin A and genistein, wherein the ratio of biochanin A to genistein is 9:1, and the composition comprises a dosage of total phytoestrogens ranging from 1 to 10 mg, wherein the concentration of biochanin A is 9 μM and the concentration of genistein is 1 μM.

**5**. The method of claim **4**, wherein the composition comprises the dosage of total phytoestrogens no more than 10 mg.

**6**. The method of claim **4**, wherein said composition enhances osteoblast differentiation.

**7**. A method of treating osteoporosis in a subject, comprising the step of administering to the subject in need thereof a composition comprising biochanin A and genistein, wherein the composition provides the subject biochanin A and genistein at a ratio of 9:1, and the composition is formulated as intravenous dosage forms, said composition comprises a dosage of total phytoestrogens ranging from 1 to 10 mg, wherein the concentration of biochanin A is 9 μM and the concentration of genistein is 1 μM.

**8**. The method of claim **7**, wherein the composition is formulated in the form of injection, solution, or suspension.

**9**. The method of claim **7**, wherein the composition comprises a dosage of total phytoestrogens less than 4 to 8 mg.

**10**. The method of claim **7**, wherein the composition comprises the dosage total phytoestrogens no more than 10 mg.

* * * * *